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A STUDY OF NONSENSE REGULATORY MUTATIONS AFFECTING METHIONINE BIOSYNTHESIS IN *Escherichia coli*

by

© HANNA L. MOROWICZ

A THESIS

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FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled A study of nonsense regulatory mutations affecting methionine biosynthesis in *Escherichia coli* submitted by Hanna L. Morowicz in partial fulfilment of the requirements for the degree of Master of Science.

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ABSTRACT

Two hundred and five spontaneous mutants of *Escherichia coli* resistant to the methionine analogue ethionine were isolated. These mutants were screened for the presence of *amber* mutations by lysogenization with $\phi 80 psu3^{+}$. Forty-one of the mutants became ethionine-sensitive in the presence of the *amber* suppressor, and were classified as presumptive *amber* mutants.

Transduction mapping with phage P1 revealed three distinct classes among these *amber* mutants. The first class consisted of *metJ* mutants. They were all closely linked to the *metB* gene and displayed significantly derepressed levels of β-cystathionase (5-14 fold) and ATP:L-methionine *S*-adenosyltransferase (2-3 fold), thus confirming that the *metJ* gene product is involved in repression of methionine biosynthesis. Of the ten presumptive *amber metJ* mutants, three (*65a*, *89b*, and *93b*) showed wild type enzyme levels in the presence of the *amber* suppressor. The existence of these mutants provides clear evidence that the *metJ* gene product is a protein which acts to repress methionine biosynthesis. One of the *metJ amber* mutants (*65a*) was found to be trans-dominant in the presence of a wild type *metJ* allele, indicating that the repressor protein is probably oligomeric in structure.

The second class included the *metK* mutants, all of which were cotransducible with the *serA* locus and showed low ATP:L-methionine *S*-adenosyltransferase activity. In most of the *metK* mutants the reduction in ATP:L-methionine *S*-adenosyltransferase activity was

accompanied by a 2-3 fold increase in the levels of β-cystathionase indicating that either the *metK* enzyme itself, or the product of its enzymatic reaction (*S*-adenosylmethionine) participates in methionine regulation. Presence of the *amber* suppressor restored normal levels of ATP:L-methionine *S*-adenosyltransferase in four of these mutants. The residual *metK* enzyme activity in two *amber* mutants *viz.*, *metK1152* and *metK1168* in the absence of the suppressor was only 2% or less and yet their growth rates appeared to be unaffected. This seems to suggest that either there is an alternate route for the synthesis of *S*-adenosylmethionine, or that a different donor is available for methylation.

To the third class belonged mutants whose ethionine-resistance did not appear to be directly related to methionine regulation. These mutants did not map at the *metJ* or *metK* loci and their levels of β-cystathionase and ATP:L-methionine *S*-adenosyl-transferase were not appreciably affected. The methionine transport system as well as the extent of RNA methylation appeared to be normal. Most probably ethionine-resistance in these mutants is caused by degradation or modification of the analogue rather than by acquisition of a defect in regulation.

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TABLE OF CONTENTS

CHAPTER	PAGE
INTRODUCTION AND LITERATURE REVIEW	1
MATERIALS AND METHODS	11
Bacterial and phage strains	11
Media	11
Isolation of ethionine-resistant mutants	11
Screening procedure to identify *amber* mutants	13
Genetic mapping	14
Selection of spontaneous su^{+}_{am} derivatives of ethionine-resistant mutants	14
Dominance test	14
Testing for *amber* mutants in the *metK* gene using polarity suppressor *suA*	15
Growth of bacteria for enzyme assays and preparation of cell extracts	15
Methylation assay	17
Methionine uptake assays	18
RESULTS	19
Isolation and classification of *amber* mutants	19
metJ amber mutants	19
metK amber mutants	27
Other *amber* ethionine-resistant mutants	33
DISCUSSION	42
BIBLIOGRAPHY	49

LIST OF TABLES

TABLE		PAGE
1	Bacterial strains	12
2	Mapping of *metJ* mutants	20
3	Identification of true *amber* mutations among various apparent *amber metJ* mutants by comparison of enzyme activities in the presence or absence of an suppressor (ϕ80p*su3*$^+$)	22
4	Restoration of repression of *metB* and *metF* biosynthetic enzymes in *metJ amber* mutants carrying ϕ80p*su3*$^+$	23
5	Comparison of enzyme activities of some *metJ* mutants with their *su*$^+$ derivatives of spontaneous origin	25
6	Dominance test for *metJ* mutants	26
7	Mapping of *metK amber* mutants	28
8	Enzyme activities of CSH4-derived *metK* mutants in the presence and absence of ϕ80p*su3*$^+$	29
9	The levels of β-cystathionase and *S*-adenosyltransferase in NR274-derived *metK* mutants in the presence and absence of ϕ80p*su3*$^+$	30
10	Specific activities of *metK* mutants and their *su*$^+$ derivatives of spontaneous origin	32
11	β-Cystathionase and *S*-adenosyltransferase levels in CSH4-derived *amber* ethionine-resistant mutants unlinked to *metB* or *serA* loci	34
12	Methyl acceptor activity of bulk RNA from *amber* ethionine-resistant mutants unlinked to *metB* or *serA* loci	36
13	Uptake of ^{14}C-methionine by wild type CSH4 cells in the presence of culture supernatant from various ethionine-resistant mutants	41

LIST OF FIGURES

FIGURE		PAGE
1	The pathway for biosynthesis and utilization of methionine in *E. coli*	4
2	Linkage map of *E. coli* showing the location of the structural and regulatory genes of methionine biosynthesis.	5
3	Comparison of ^{14}C-methionine uptake by wild type cells with the uptake by *amber metK* mutants (*1018*, *1152*, *1154*) and ethR*1038* mutant.	38
4	Time course of ^{14}C-methionine uptake by wild type cells and by *amber* mutants *viz.*, *metK1018*, *1152*, *1154*, and ethR*1038* in the presence of ϕ80p*su3*$^+$.	39

INTRODUCTION AND LITERATURE REVIEW

The conceptual framework for studies on the expression of structural genes and the regulation of this expression was provided by the operon model of Jacob and Monod (1961). The basic features of this model involve the presence of a cluster of genes controlling related functions, which are transcribed as one polycistronic messenger RNA and are regulated together. The regulation of the operon is accomplished by two elements, the first one being the regulatory signal (repressor and/or activator) produced by one or more genes not necessarily adjacent to the structural genes, and the second - a site upon which the signal acts (the operator) located at the beginning of the operon. The affinity of the repressor or activator molecule for its operator site would be influenced by the presence of either the metabolic end product or the substrate, thus explaining the well recognized phenomenon of genetic adaptation.

This model was based mainly on the evidence from studies of lactose utilization in *Escherichia coli*. However, its broad applicability (with slight modifications) to a variety of anabolic and catabolic systems in procaryotes has been widely documented. Systems that apparently do not conform to the operon model because of lack of linkage of the functionally related genes still fulfil its major criterium of being regulated together by a common controlling element. This element must now interact with individual recognition sites (operators) located adjacent to each structural gene, and since the affinity of the repressor for these sites may not be the same,

non-coordinate regulation is expected.

The study of regulation of gene expression originated and developed with the isolation of regulatory mutants. It is because of these mutants that the involvement of various controlling elements and the sites of their action could be recognized. Since according to the original Jacob-Monod model the regulatory elements would have no other function in the cell, some of the regulatory mutants would not produce an easily recognizable phenotype and therefore they would be difficult to detect. A method for the identification and characterization of regulatory genes using metabolite analogues was first introduced by Cohen and Jacob in 1959. The mutants of *E. coli* that they investigated were resistant to 5-methyltryptophan, contained elevated levels of tryptophan biosynthetic enzymes, and mapped outside of the tryptophan operon at a locus which is now referred to as the structural gene for the tryptophan repressor (*trpR*). The same analogue was used by Moyed (1960) to obtain different kinds of regulatory mutants which became insensitive to feedback inhibition by tryptophan, overproduced the metabolite and mapped in one of the structural genes. The regulation of histidine biosynthesis in *Salmonella typhimurium* was also elucidated using various histidine analogues. Triazolealanine was used to select mutants derepressed for histidine biosynthesis which mapped in several loci, all of which were shown to participate in formation or modification of histidinyl-tRNA^{His}, thus implicating this molecule in repression (Roth and Hartman, 1965; Roth *et al.*, 1966). Mutants unable to carry out normal regulatory functions were also isolated in the arginine system employing the structural analogue canavanine which competes with

arginine in protein synthesis (Maas, 1961). These mutants were shown to have elevated levels of arginine biosynthetic enzymes and to be insensitive to both arginine and canavanine repression. These are just a few examples of the extensive use of analogues in the investigation of the genetic and biochemical mechanisms of regulation. A comprehensive review of this subject can be found in an article by Umbarger (1971).

The regulation of methionine biosynthesis has also been investigated with the aid of analogue-resistant mutants. Since methionine is involved in several different metabolic activities such as the initiation and assembly of proteins, as donor of the propylamine moiety in polyamine biosynthesis, and as the principal methyl donor via *S*-adenosylmethionine, the regulatory mechanism for its biosynthesis would be expected to be more complex than that for other amino acids. A detailed review of the genetic and biochemical aspects of methionine biosynthesis has been published by Smith (1971). The essential features of this biosynthesis are outlined below.

Six structural genes participate in the conversion of homoserine to methionine (Fig. 1). These are clustered in non-contiguous segments on the *E. coli* chromosome (Fig. 2), *metA* and *metH* being located at 79.5 min, *metB* and *metF* at 78 min, *metC* at 55.5 min, and *metE* at 75.5 min.

The first specific precursor of methionine, *O*-succinylhomoserine, is formed from succinyl-CoA and homoserine by the enzyme *O*-succinylhomoserine synthetase (*metA*). The next enzyme, cystathionine-γ-synthetase (*metB*) catalyzes the replacement of succinyl group by cysteine to give cystathionine. Purified *metB* enzyme has been found to consist

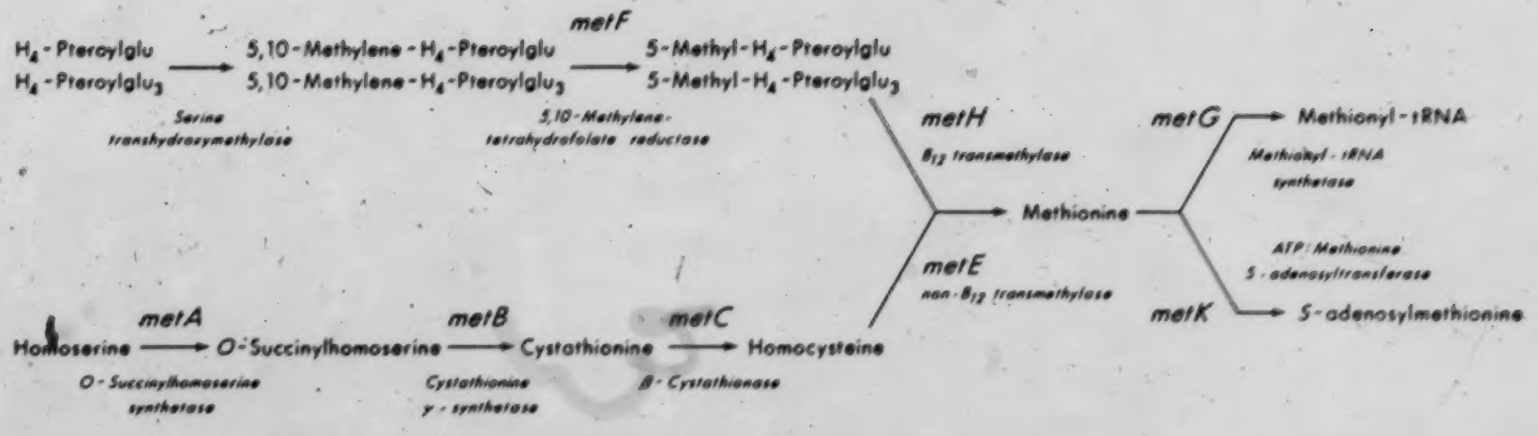

Figure 1. The pathway for biosynthesis and utilization of methionine in *E. coli*

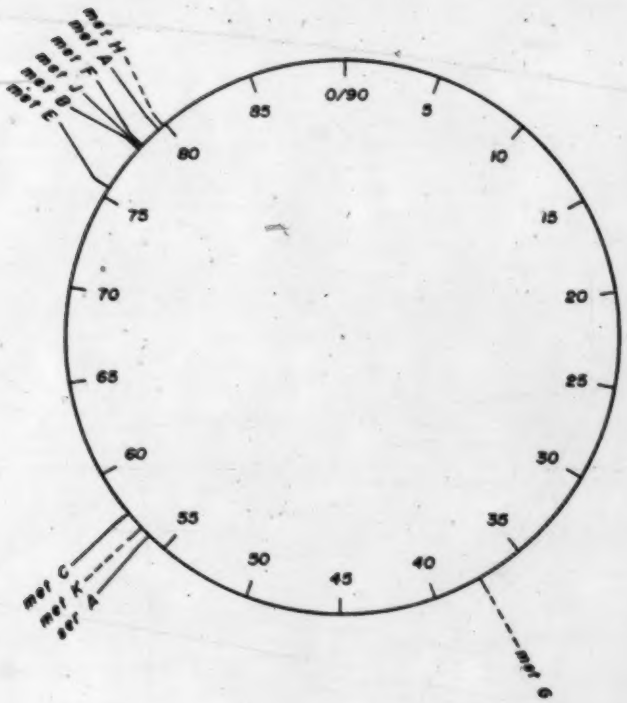

Figure 2. Linkage map of *E. coli* showing the location of the structural and regulatory genes of methionine biosynthesis. Broken lines indicate approximate locations of the genes.

of four identical subunits each with a molecular weight of 40,000. Cystathionine is then hydrolyzed to homocysteine by the *metC* enzyme, β-cystathionase. The methyl group donors for the methylation of homocysteine to methionine are synthesized by the enzyme 5,10-methylene-tetrahydrofolate reductase (*metF*), and the actual methylation of homocysteine is carried out by either the vitamin B_{12}-independent transmethylase (*metE*) or the B_{12}-dependent enzyme (*metH*).

Once methionine is formed in the cell it will serve either in protein synthesis or in the synthesis of *S*-adenosylmethionine (SAM). The enzyme which aminoacylates the two species of $tRNA^{Met}$, methionyl-tRNA synthetase, is coded by the *metG* gene. The formation of *S*-adenosylmethionine from methionine and ATP is catalyzed by ATP:L-methionine *S*-adenosyltransferase (also referred to as the *metK* enzyme). The function and properties of this enzyme are described in review articles by Lombardini and Talalay (1971) and by Mudd (1972). The *S*-adenosyltransferase from *E. coli* cannot utilize the methionine analogue, ethionine, as a substrate, unlike the yeast and rat liver enzymes. The principal metabolic functions of *S*-adenosylmethionine are participation in polyamine biosynthesis (Tabor *et al.*, 1961) via decarboxylated SAM, and in various transmethylation reactions (Cantoni, 1965).

As in many other biosynthetic pathways, synthesis of methionine biosynthetic enzymes is repressed by the addition of the end product of the pathway. This repression has been shown to be non-coordinate in both *S. typhimurium* (Lawrence *et al.*, 1968) and *E. coli* (Holloway *et al.*, 1970). The regulation of methionine enzyme levels was first investigated in *S. typhimurium* by Lawrence *et al.* (1968). Three

genes (*metI*, *metJ*, and *metK*) were found to be altered in mutants resistant to the methionine analogues ethionine, α-methylmethionine, and norleucine.

The *metI* mutants which were resistant to α-methylmethionine and ethionine were found to map within the *metA* gene coding for the first biosynthetic enzyme. It has been demonstrated by Lee *et al.* (1966) that the *metA* enzyme is subject to strong cooperative feedback inhibition by methionine and *S*-adenosylmethionine. Thus it appeared that the *metI* mutants were feedback-insensitive and since no complementation between them was observed (Smith, 1971), it was concluded that the enzyme must consist of one polypeptide chain with regions determining the catalytic properties and regions involved in regulation. All of the *metI* mutants examined excreted methionine, but had normal levels of the biosynthetic enzymes and were subject to repression by methionine.

The *metJ* mutants were resistant to inhibition by ethionine and mapped close to the *metB* locus. They were shown to exhibit constitutive or derepressed synthesis of all the methionine biosynthetic enzymes and of the *metK* enzyme. Further studies (Su and Greene, 1971; Chater, 1970) of the nature of the *metJ* mutations proved that this gene exerts its effect via a diffusible product since partial diploids heterozygous for the *metJ* allele were found to be repressible by methionine. Minson and Smith (1972) obtained *metJ* mutants in *Salmonella* which were suppressible by *amber*-suppressor, thus suggesting that the *metJ* gene codes for a protein. However, their only criterium for suppression was the phenotype on medium containing ethionine and no enzyme activities

were reported. The *metJ* mutation has been found not to have any significant effect on the synthesis or modification of $tRNA^{Met}$ (Ahmed, 1973); however, the free amino acid pool appeared to be affected (Clandinin and Ahmed, 1973). For instance, in some strains the level of threonine and histidine was increased tenfold as compared to the wild type.

The third distinct class of regulatory mutants, *metK*, was resistant to ethionine, α-methylmethionine and norleucine. These mutants were shown to be defective in the *S*-adenosyltransferase (Hobson and Smith, 1973; Greene *et al.*, 1970). Some of these strains had derepressed levels of methionine enzymes and excreted methionine, others were non-excretors and repressible by methionine. The non-excretors produced *metK* enzyme with an increased *Km* for methionine, whereas the excretors most probably produced an entirely defective enzyme since formation of SAM was undetectable. One of the excreting *metK* mutants was found to have unusual characteristics (Hobson, 1974) in that it had normal levels of *S*-adenosyltransferase activity and complemented a number of other *metK* mutants.

In view of these findings it appears that the *metJ* gene codes for a regulatory element (most likely a repressor protein) capable of recognizing operator sequences linked to the structural genes for methionine biosynthesis. The nature of the corepressor is still poorly understood but the evidence seems to indicate that either the product of the *metK* gene (SAM) or the *metK* enzyme itself is involved in repression. However, since bacterial cells are impermeable to SAM, it is not possible to obtain direct evidence. Also, the role of methionine in repression and the nature of its interaction with the aporepressor remains unresolved.

Consequently, it appeared desirable to re-examine the nature of methionine regulatory mutants with the hope that they may point to new, unrecognized genetic elements and reveal biochemical interactions not yet detected. For this purpose *amber* mutants resistant to the methionine analogue, ethionine, were isolated and studied. Ethionine causes growth inhibition probably because it is incorporated into proteins instead of methionine (Spizek and Janecek, 1969). Theoretically different kinds of mutations could confer ethionine-resistance, such as (1) altered regulatory elements resulting in increased internal pools of methionine effectively competing with the analogue, (2) mutations leading to blockage in methionine utilization, thus causing accumulation of the metabolite, (3) altered methionyl-tRNA synthetase or $tRNA^{Met}$ incapable of recognizing ethionine, (4) defective uptake system, and (5) efficient degradation of the analogue. Since only *amber* mutants were selected they must have arisen in genes whose functions were dispensable and which coded for protein products. Also, if any of these genes were located within an operon or operons, polar effects would have been detectable. Especially important was the biochemical investigation of *amber metJ* mutants since those reported in *Salmonella* were characterized on the basis of ethionine phenotype only, thus giving rise to speculations concerning the nature of the *metJ* gene product. Nevertheless, even the existence of *amber metJ* mutants would not be sufficient to implicate the *metJ* product as the repressor protein since the gene product may catalyze the synthesis of an active repressor. The isolation of *amber metJ* mutants, however, may aid in the eventual purification of the protein for use in an *in vitro* system to study

regulation. Only then could sufficient evidence be provided to elucidate the exact nature of the corepressor(s) and its interaction with the repressor.

MATERIALS AND METHODS

Bacterial and phage strains

A list of the strains used is given in Table 1. All strains were derived from *E. coli* K12. P1*vir*, the generalized transducing phage, was obtained from Dr. A. Ahmed's collection. Phage ϕ80p*su3*$^+$/*su3*$^-$*h*$^-$, used for testing of *amber* suppression, was supplied by Dr. N. Franklin.

Media

The minimal medium used was that of Davis and Mingioli as described by Roth (1970). Required amino acids were supplemented at a concentration of 20 mg/l and thiamine HCl at a concentration of 10 mg/l. L broth was used as the complete medium.

Isolation of ethionine-resistant mutants

NR274 was used as the parental strain for isolation of ethionine-resistant mutants of the *1-100* series. Single colonies were grown to saturation in L broth and then 0.01-0.02 ml of the culture was spread with 0.1 ml pipette on a 1/8 sector of a glucose minimal plate supplemented with tryptophan and containing 3 g/l of DL-ethionine. In this way eight different cultures could be spread on one plate. Plates were incubated at 37° for 48 hrs, after which time a few single colonies appeared along each streak. Two single colonies were picked from each culture (or sector of a plate) and taken through two single colony purifications on the same medium. Stocks of these mutants were maintained on minimal medium with and without ethionine. It was observed

TABLE 1 - Bacterial Strains

Strain	Genotype	Source
CSH4	F⁻ *lacZ*$_{am}$ *trp*$_{am}$ *strA*	Cold Spring Harbor strain kit
HM *1016-1174* series	F⁻ *lacZ*$_{am}$ *trp*$_{am}$ *strA* ethR	DL-ethionine-resistant derivatives of CSH4
NR274	HfrC *lacZ*$_{am}$ *trp*$_{am}$	N. Rutter (P1 sensitive derivative of CA274)
HM *1-100* series	HfrC *lacZ*$_{am}$ *trp*$_{am}$ ethR	DL-ethionine-resistant derivatives of NR274
AT2475	HfrH *serA thi su*$^0_{am}$	A. Ahmed stock collection, U. of Alberta
HfrR1	Hfr *metB rel su*$^0_{am}$	B. Bachmann, Yale University
AB1206	F'14/Δ(*ilv met arg*) *proA his thi lacY galK strA tfr*	B. Bachmann, Yale University
MA200*ilv*	F⁻ *ilv serA trp his strA* λ^R *su*$^0_{am}$	derived from Ma220 by transduction to argA⁺ and EMS mutagenesis to ilv⁻
R2	F⁻ *mtl malA ilv glpK metB argH thi strA su*$^0_{am}$	E. Holowachuk (from Lin 161), U. of Alberta
333*suA2*	*thr trpE9851 suA*	P1 sensitive derivative of 333*suA*
X341	HfrC *metB pro thi rel*	A. Ahmed stock collection, U. of Alberta
GM48	*dam-3 dcm-6 gal1 gal2 ara leu thr lac ton tsx*	A. Ahmed stock collection, U. of Alberta

that mutants kept on L broth plates for few generations tended to lose their ethionine-resistant phenotype.

Ethionine-resistant mutants of the *1016-1174* series were isolated from strain CSH4. Essentially the same method was used as that employed in isolating the mutants of the *1-100* series except that 0.1 ml of each overnight culture was spread on one plate containing 7 g/l of DL-ethionine. Plates were incubated at 30° for 48-72 hrs, and one colony from each plate was taken for further purification.

Each of the ethionine-resistant mutants came from a separate overnight culture to ensure that each represented an independent mutational event.

Screening procedure to identify amber mutants

Phage $\phi 80psu3^+$ was used in screening for *amber* ethionine-resistant mutants. This phage carries suppressor $tRNA_I^{Tyr}$ which efficiently suppresses the *lac* and *trp amber* mutations in NR274 and CSH4.

A lysate of this phage was spread on glucose minimal plates (lacking tryptophan) which were then streaked with the ethionine-resistant mutants and incubated at 30° for 24-36 hrs. If lysogenization had occurred, a thick growth on each streak was visible. Faint growth usually indicated lack of lysogenization and was probably due to the broth in which phage was suspended. Each lysogen was purified on minimal glucose plates and then tested for growth on lactose minimal medium. The mutants which simultaneously acquired lac^+ trp^+ phenotype were considered to be lysogenic for $\phi 80psu3^+$. Next they were tested for ethionine inhibition on minimal glucose plates containing DL-ethionine at 3 g/l (NR274 mutants,

incubated at 37°) or 7 g/l (CSH4 mutants, incubated at 30°). Growth was scored after 48 hrs of incubation.

If the mutation leading to ethionine-resistance was of the *amber* type, presence of an *amber* suppressor would be expected to change the phenotype from ethionine-resistance to sensitivity. Therefore, those mutants which became sensitive to ethionine upon lysogenization were classified as *amber* ethionine-resistant mutants.

Genetic mapping

Generalized transducing phage P1*vir* was used for mapping according to the procedure described by Lennox and Yanofsky (1959).

Selection of spontaneous su^+_{am} *derivatives of ethionine-resistant mutants*

Spontaneous su^+ derivatives for some of the NR274 *1-100* mutants were obtained by plating 0.1-0.2 ml of overnight cultures on lactose minimal plates, followed by incubation at 30° or 37° for 3 days. The only colonies that could grow were those with the lac^+ trp^+ phenotype. Presence of su^+ in these strains was confirmed using a T4 *amber* mutant which will give rise to plaques only on strains carrying *amber* suppressors.

Dominance test

The R2 strain was used as a recipient for transduction to $metB^+$ by P1*vir* grown on several *metJ 1-100* mutants. The met^+ transductants were tested for ethionine-resistance (cotransduction of *metJ* allele with $metB^+$ allele) and one ethionine-resistant transductant from each P1 lysate was purified. Next the R2 *metJ* strains carrying different *metJ* alleles

were used as recipients in mating with F'14 (AB1206). Spot mating was performed in which the donor was grown to early log phase and the recipients to mid-log phase. A drop of each recipient was placed on glucose minimal B_1 plate. When the spots were dry a drop of the donor was pipetted on top of them. Confluent growth was obtained from each mating. The recombinants carrying F'14 were purified by streaking for single colonies and twenty-five single colonies per mating were streaked on glucose minimal B_1 plates. These were then tested for resistance to DL-ethionine (3 g/l) at 37° and scored after 48 hrs of incubation.

Testing for amber mutants in the metK gene using polarity suppressor suA

Strain Ma220*ilv* was transduced to $serA^+$ with P1 lysates grown on *metK1152* and *metK1168*. Transductants were scored for ethionine-resistance. Two ethionine-resistant (*metK*) transductants from each strain were purified. These were used as recipients in transduction to ilv^+ with P1 grown on 333*suA2*. Two hundred ilv^+ colonies per strain were purified and tested for ethionine-resistance.

Growth of bacteria for enzyme assays and preparation of cell extracts

Cells were grown to stationary phase in 3 ml minimal medium containing the required amino acids. This culture was then transferred into 20 ml of the same medium and incubated overnight. The following day cells were harvested by centrifugation and inoculated into 200 ml of fresh minimal medium. After 3-4 hrs of growth, cultures were centrifuged, washed in saline, and stored at -40°.

On several occasions this procedure was modified as follows:

5 ml minimal saturated cultures were centrifuged, inoculated into 50 ml fresh minimal medium and incubated until late log phase.

For extract preparations, cell pellets were suspended in the appropriate buffer (depending on the enzyme assay to be performed), sonicated twice for 30 sec in ice, and then centrifuged at 31000 g for 30 min in a Sorval RC2-B centrifuge. These crude extracts were used directly without freezing in all enzyme assays. All enzymes except ATP:L-methionine *S*-adenosyltransferase were found to be stable for at least 24 to 48 hrs.

Cystathionine-γ-synthetase was assayed by measuring the amount of α-ketobutyrate formed as described by Kaplan and Flavin (1966). The specific activity is expressed as decrease in absorbance at 340 nm per 20 min per mg protein at 37°.

β-*Cystathionase* was assayed by the procedure of Flavin (1962). The specific activity is described as increase in absorbance at 412 nm per min per mg protein at room temperature.

5,10-Methylenetetrahydrofolate reductase was determined by the menadione-dependent back reaction described by Dickerman and Weissbach (1964). The specific activity is described as nmoles formaldehyde formed in 30 min per mg protein at 37°.

ATP:L-methionine S-adenosyltransferase was assayed in the *1016-1174* series mutants by the procedure of Tabor and Tabor (1971). The amount of enzyme per assay was from 50 to 100 μg protein. ^{14}C-Methionine (20 μCi/μM) at a final concentration of 175 μM per assay was used. The ^{14}C-adenosylmethionine was separated from ^{14}C-methionine by TLC in 95% ethanol, acetic acid, water (80:5:15) solvent and counted

in toluene-Omnifluor scintillation mixture. The specific activity is described as nmoles *S*-adenosylmethionine formed in 30 min per mg protein at 37°.

The mutants of the *1-100* series were assayed using the same reaction mixture except the final concentration of ^{14}C-methionine (5 µCi/mM) was 500 µM per assay and the extracts were prepared not in Nirenberg buffer (Nirenberg and Matthaei, 1961), but in 0.05 M phosphate buffer, pH 7.3. The radioactive product ^{14}C-SAM was separated from the substrate ^{14}C-methionine using BioRex-70 (50-100 mesh) resin according to the method described by Holcomb and Shapiro (1975) and modified by C. Somerville (unpublished). The specific activity is expressed as µmoles *S*-adenosylmethionine formed in 20 min per mg protein at 37°.

Protein concentration was estimated using Folin-Ciocalteu reagent or by Biuret reaction as described by Layne (1957).

Methylation assay

Cells grown to stationary phase in L broth were used to extract bulk RNA. To 1 g of cells suspended in 2 ml buffer (0.001 M Tris at pH 7.4 containing 0.01 M Mg-acetate), 2 ml water-saturated phenol were added and agitated on shaker in ice for 1 hr. The mixture was centrifuged for 30 min at 30000 g and the aqueous layer removed. RNA was precipitated at -30° overnight by adding 0.1 volume of 20% potassium acetate and 2 volumes of 95% ethanol (at -20°). The precipitate was collected by centrifugation at 31000 g for 10 min and washed twice with cold 75% ethanol. After washing, the precipitate was dissolved in 0.2 ml water, centrifuged to remove undissolved material and stored at -20°.

The average yield of RNA was approximately 50-60 A_{260} units per gm of cells.

Preparation of methylating enzyme from strain CSH4 was carried out according to the method of Marinus and Morris (1973).

Methyl-deficient RNA was prepared from strain X341 grown under conditions described by Fleissner and Borek (1966).

The reaction mixture was that of Hurwitz and Gold (1966). 8 µM (1 µCi) of (methyl-^{3}H)-*S*-adenosylmethionine, 1-2 A_{260} units RNA and 500 µg protein (methylating enzyme extract) were used per assay. The reaction was stopped by precipitation with cold 5% TCA (see Fleissner and Borek, 1966), filtered through Whatman GF/C glass fiber filters, washed with 5x5 ml 5% TCA and counted in a toluene-Omnifluor scintillation mixture. Methyl-acceptor activity of RNA is described as nmoles of methyl group transferred in 15 min per A_{260} unit of RNA.

Methionine uptake assays

Experiments were carried out according to the procedure described by Kadner (1974). The cell concentration was adjusted to A_{420} of 1.0, and viable counts were made. ^{14}C-Methionine was used at a concentration of 0.8 µM (0.05 µCi) per assay. The rate of uptake is expressed as pmoles ^{14}C-methionine transported per 10^8 viable cells.

RESULTS

Isolation and classification of amber mutants

Forty-five independent ethionine-resistant mutants were isolated from strain NR274 HfrC *trp*$_{am}$ *lac*$_{am}$. Seventeen of them (38%) became ethionine-sensitive when lysogenized with ϕ80p*su3*$^+$. This was the basis for their classification as ethionine-resistant *amber* mutants. In strain CSH4 F$^-$ *trp*$_{am}$ *lac*$_{am}$, one hundred and sixty independent mutants were screened for *amber* mutations and twenty-four of them (15%) fell into this category.

In order to determine the location of the *amber* mutants P1*vir* lysates for all these mutants were prepared and used to transduce strain AT2475 to serA$^+$ and HfrR1 to metB$^+$. The analysis of the transductants revealed three classes among the *amber* mutants. The *metJ* mutants belonged to the first class, the *metK* mutants belonged to the second class, and the third class consisted of mutants which were neither *metJ* nor *metK*. None of the latter mutants were of the *metI* type since ethionine-resistance was not cotransductible with either the *metA* or the *argH* genes.

metJ amber mutants

P1 lysates were grown on all of the mutants classified as *amber* ethionine-resistant on the basis of ϕ80p*su3*$^+$ test. Fifty *metB*$^+$ transductants from each P1 lysate were purified and tested for resistance to ethionine. Table 2 lists the mutants identified as *metJ* and gives the cotransduction frequencies between *metB* and *metJ* alleles and between

TABLE 2

Mapping of *metJ* mutants

Strain	% cotransduction of ethionine-resistance with *metB*	% cotransduction of ethionine-resistance with *argH*
wild type NR274	0	0
metJ64b	98	26
metJ65a	94	20
metJ71a	92	19
metJ72b	98	27
metJ76a	98	28
metJ78b	98	28
metJ85a	96	27
metJ87a	98	28
metJ89b	92	20
metJ93b	98	29

argH and *metJ* alleles. All of the *metJ* mutants were found in the *1-100* series derived from strain NR274.

Previously *metJ* mutants have been shown to exhibit derepressed levels of methionine biosynthetic enzymes. In order to confirm the mapping results, each of the mutants was assayed for β-cystathionase and ATP:L-methionine *S*-adenosyltransferase activities in both the suppressed (φ80p*su3*$^+$ lysogens) and unsuppressed state. As presented in Table 3 all of the strains tested showed constitutive synthesis for both enzymes in the absence of the suppressor. Contrary to expectations, most of the suppressed mutants still showed elevated levels of enzymes even though they had become fully ethionine-sensitive. Only three strains (*65a*, *89b* and *93b*) show complete suppression of their original *metJ* mutation by the *amber* suppressor *su3*$^+$, and therefore can be unequivocally classified as *ambers*. Comparison of the activities of two other methionine biosynthetic enzymes (Table 4) confirms the classification of these three mutants as *ambers* by their efficient suppressibility.

Since some of the *metJ* mutants previously identified as *amber* (on the basis of the ethionine plate test) showed only partial decrease in specific activities in the presence of φ80p*su3*$^+$ *amber* suppressor, spontaneous *su*$^+$ derivatives were obtained in order to eliminate the possible interference by φ80 phage with ethionine uptake. The *su*$^+$ derivatives were selected as simultaneous revertants to the trp$^+$ lac$^+$ phenotype and tested for supporting the growth of T4 *amber*. Approximately ten *su*$^+$ revertants for each of three different apparent *amber metJ* strains were tested for sensitivity to ethionine; however the response was always non-uniform, i.e. among the *su*$^+$ revertants for a mutant

TABLE 3 - Identification of true *amber* mutations among various apparent *amber metJ* mutants by comparison of enzyme activities in the presence or absence of an *amber* suppressor (ϕ80p*su3*$^+$)

Strain	Relative specific activities		Identification
	β-cystathionase	ATP:L-methionine *S*-adenosyltransferase	
wild type NR274	1.0[a]	1.0[c]	
NR274 (ϕ80p*su3*$^+$)	1.0[b]	1.0[d]	
64b	16.5	2.74	
64b (ϕ80p*su3*$^+$)	10.7	2.7	
65a	6.66	2.2	*amber*
65a (ϕ80p*su3*$^+$)	1.09	0.92	
71a	11.59	2.52	
71a (ϕ80p*su3*$^+$)	9.63	2.0	
72b	5.2	2.23	
72b (ϕ80p*su3*$^+$)	3.2	1.81	
76a	9.15	3.34	
76a (ϕ80p*su3*$^+$)	4.11	2.21	
78b	10.1	2.96	
78b (ϕ80p*su3*$^+$)	11.12	2.91	
85a	12.1	2.33	
85a (ϕ80p*su3*$^+$)	9.5	1.7	
87a	13.9	2.35	
87a (ϕ80p*su3*$^+$)	6.34	1.61	
89b	11.87	2.9	*amber*
89b (ϕ80p*su3*$^+$)	0.96	0.98	
93b	10.55	2.82	*amber*
93b (ϕ80p*su3*$^+$)	1.05	0.99	

The enzyme activities of the mutants are expressed relative to wild type which is taken as 1.0. The activities of the ϕ80p*su3*$^+$ lysogens are expressed relative to NR274 (ϕ80p*su3*$^+$) enzyme level which is taken as 1.0.

a. Increase in absorbance at 412 nm of 0.138 per min per mg protein.
b. Increase in absorbance at 412 nm of 0.178 per min per mg protein.
c. 10.5 µmoles *S*-adenosylmethionine formed in 20 min per mg protein.
d. 13.1 µmoles *S*-adenosylmethionine formed in 20 min per mg protein.

TABLE 4

Restoration of repression of *metB* and *metF* biosynthetic enzymes in *metJ amber* mutants carrying $\phi 80 psu3^+$

Strain	Relevant genotype	Relative specific activities	
		Cystathionine-γ-synthetase (*metB*)	5,10 methylene-tetrahydrofolate reductase (*metF*)
NR274	$metJ^+$	1.0[a]	1.0[b]
NR274 ($\phi 80 psu3^+$)		1.05	1.0
51b	*metJ non-amber*	2.18	4.69
51b ($\phi 80 psu3^+$)		2.25	6.83
65a	*metJ amber*	1.8	3.76
65a ($\phi 80 psu3^+$)		0.6	0.65
89b	*metJ amber*	2.46	4.1
89b ($\phi 80 psu3^+$)		0.72	1.0
93b	*metJ amber*	4.94	4.52
93b ($\phi 80 psu3^+$)		0.97	0.98

The enzyme activities of the mutants and their lysogens are expressed relative to the wild type level which is taken as 1.0.

a. Decrease in absorbance at 340 nm of 1.9 in 20 min per mg protein.

b. 0.24 nmole formaldehyde formed in 30 min per mg protein.

there were both ethionine-sensitive and resistant colonies. This was interpreted to mean that some of the *su*$^+$ revertants acquired suppressor tRNAs which inserted amino acids not capable of restoring wild type *metJ* activity thus producing ethionine-resistant phenotype. Those *su*$^+$ revertants which were ethionine-sensitive retained their original *metJ* mutation which could be transduced into another strain. Consequently these ethionine-sensitive *su*$^+$ strains were used to compare their enzyme levels with those of the original mutants. The results are shown in Table 5. Only in the case of strain *71a su*$^+$ could the ethionine-sensitive phenotype be explained in terms of considerable decrease in enzyme activities. *Su*$^+$ derivatives of the other two apparent *amber metJ* mutants (*78b* and *85a*), although also ethionine-sensitive on plates, displayed no change in enzyme levels.

All of the *metJ* mutants isolated thus far have been found to be recessive to the wild type allele. The only exception is a *Salmonella* mutant cited by Chater (1970) which in a partial diploid state showed slightly elevated enzyme activity as compared to the wild type. Therefore the *metJ* mutants derived from NR274 were examined for their dominance. Recipient strains carrying various *metJ* alleles were mated with F'14 which contains a wild type copy of the *metJ* gene. Recombinants were selected on minimal medium so that only those colonies which received F'14 would become ilv$^+$ and arg$^+$ and would grow. These merodiploids were then tested for ethionine-sensitivity and five of them (four *metJ amber* mutants and one *53b non-amber*) displayed full ethionine-resistance suggesting the possibility that these were dominant mutations. Table 6 compares the enzyme activities of these partial diploids with

TABLE 5

Comparison of enzyme activities of some *metJ* mutants with their *su*$^+$ derivatives of spontaneous origin

Strain	Relevant genotype	Relative specific activities	
		β-cystathionase	ATP:L-methionine *S*-adenosyltransferase
NR274	*metJ*$^+$ ethS	1.0[a]	1.0[b]
NR274 *su*$^+$	*metJ*$^+$ *su*$^+$ ethS	1.0	1.0
71a	*metJ* ethR	14.1	2.88
71a su$^+$	*metJ su*$^+$ ethS	5.08	0.8
78b	*metJ* ethR	11.25	2.8
78b su$^+$	*metJ su*$^+$ ethS	12.67	2.77
85a	*metJ* ethR	13.0	2.33
85a su$^+$	*metJ su*$^+$ ethS	15.1	2.85

The enzyme activities are expressed relative to the wild type level which is taken as 1.0.

a. Increase in absorbance at 412 nm of 0.129 per min per mg protein.

b. 9.8 μmoles *S*-adenosylmethionine formed in 20 min per mg protein.

TABLE 6

Dominance test for *metJ* mutants

Strain	Relevant genotype	Relative specific activities	
		β-cystathionase	ATP:L-methionine *S*-adenosyltransferase
NR274		1.0[a]	1.0[c]
F'14/NR274	F *metJ*$^+$/*metJ*$^+$	1.0[b]	1.0[d]
85a		12.1	2.33
F'14/*85a*	F *metJ*$^+$/*metJ85a*	1.29	1.2
72b		5.2	2.23
F'14/*72b*	F *metJ*$^+$/*metJ72b*	2.11	1.96
71a		11.59	2.52
F'14/*71a*	F *metJ*$^+$/*metJ71a*	1.42	0.9
65a		6.66	2.2
F'14/*65a*	F *metJ*$^+$/*metJ65*$_{am}$	6.44	2.36
53b		6.3	2.13
F'14/*53b*	F *metJ*$^+$/*metJ53b*	2.25	1.78

The enzyme activities of haploid *metJ* mutants are expressed relative to the wild type (NR274) level which is taken as 1.0.

The enzyme activities of partial diploids heterozygous for *metJ* are expressed relative to enzyme level of the wild type diploid F'14/NR274 which is taken as 1.0.

a. Increase in absorbance at 412 nm of 0.138 per min per mg protein.
b. Increase in absorbance at 412 nm of 0.115 per min per mg protein.
c. 10.5 μmoles of *S*-adenosylmethionine formed in 20 min per mg protein.
d. 6.53 μmoles of *S*-adenosylmethionine formed in 20 min per mg protein.

the wild type strain also in a diploid condition. Only one strain, *65a*, is significantly derepressed and therefore certainly dominant over the wild type. Surprisingly, this is also one of the *metJ* alleles that was previously identified as an efficiently suppressible *amber*. Strains *53b* and *72b* show moderate derepression, perhaps enough to cause ethionine-resistant phenotype.

metK amber mutants

All of the apparent *amber* ethionine-resistant mutants were checked for cotransduction of ethionine-resistance with the *serA* gene in order to identify *amber metK* mutants. The transduction results (based on 100-150 colonies) are shown in Table 7. Mutants identified as *metK* were present among the *ambers* isolated from both NR274 and CSH4 strains. The cotransduction frequencies with *serA* vary from 12% to 38% for different strains. This broad range of frequencies is consistent with the observation made by Maas (1972), that depending on the size of the colonies picked, the linkage between *serA* and *metK* is anywhere from 10% (large colonies) to 50% (small colonies).

The levels of β-cystathionase and *S*-adenosyltransferase were determined in all of the *amber metK* mutants. The amount of *S*-adenosylmethionine produced was estimated by TLC for CSH4 derived mutants, and by BioRex-70 separation for NR274 mutants (as described on pages 16-17). All mutants showed reduced levels of ATP:L-methionine *S*-adenosyltransferase (Tables 8 and 9) in the presence of *amber* suppressor, thus confirming the mapping data. However, only four strains

TABLE 7

Mapping of *metK amber* mutants

Strain	% cotransduction of ethionine-resistance with *serA*
wild type NR274	0
metK41a	31
metK42b	34
metK43a	27
metK44a	38
metK45a	14
metK48a	32
metK77a	28
wild type CSH4	0
metK1018	24
metK1152	12
metK1154	18
metK1168	17

TABLE 8

Enzyme activities of CSH4-derived *metK* mutants in the presence and absence of $\phi 80psu3^+$

Strain	Relevant genotype	Relative specific activities	
		ATP:L-methionine *S*-adenosyltransferase	β-cystathionase
CSH4	*metK*$^+$	1.0[a]	1.0[b]
CSH4 ($\phi 80psu3^+$)		1.05	1.0
1042	*metK non-amber*	0.17	2.1
1042 ($\phi 80psu3^+$)		0.175	2.19
1018	*metK amber*	0.1	1.2
1018 ($\phi 80psu3^+$)		1.1	0.94
1152	*metK amber*	0.018	1.1
1152 ($\phi 80psu3^+$)		0.97	0.9
1154	*metK amber*	0.12	1.18
1154 ($\phi 80psu3^+$)		1.13	0.88
1168	*metK amber*	0.022	1.35
1168 ($\phi 80psu3^+$)		1.02	0.95

The enzyme activities are expressed relative to the wild type level which is taken as 1.0.

a. 77.2 nmoles *S*-adenosylmethionine formed in 30 min per mg protein.

b. Increase in absorbance at 412 nm of 0.17 per min per mg protein.

TABLE 9

The levels of β-cystathionase and *S*-adenosyltransferase in NR274-derived *metK* mutants in the presence and absence of φ80p*su3*$^+$

Strain	Relative specific activities	
	ATP:L-methionine *S*-adenosyltransferase	β-cystathionase
wild type NR274	1.0[a]	1.0[c]
NR274 (φ80p*su3*$^+$)	1.0[b]	1.0[d]
41a	0.22	2.9
41a (φ80p*su3*$^+$)	0.22	2.53
42b	0.37	3.4
42b (φ80p*su3*$^+$)	0.31	3.2
43a	0.34	2.4
43a (φ80p*su3*$^+$)	0.33	2.8
44a	0.34	2.35
44a (φ80p*su3*$^+$)	0.31	2.6
77a	0.29	2.9
77a (φ80p*su3*$^+$)	-	2.4

The enzyme activities of the mutants are expressed relative to wild type level which is taken as 1.0. The enzyme activities of φ80p*su3*$^+$ lysogens are expressed relative to wild type NR274 (φ80p*su3*$^+$) lysogen level which is taken as 1.0.

a. 9.8 μmoles *S*-adenosylmethionine formed in 20 min per mg protein.

b. 12.75 μmoles *S*-adenosylmethionine formed in 20 min per mg protein.

c. Increase in absorbance at 412 nm of 0.135 per min per mg protein.

d. Increase in absorbance at 412 nm of 0.18 per min per mg protein.

(all derived from CSH4) had their *metK* enzyme activity fully restored in the presence of ϕ80p*su3*$^+$ (Table 8) and could be considered true *amber* mutants. The rest of the *metK* mutants (also sensitive to ethionine when lysogenized by ϕ80p*su3*$^+$) did not exhibit any change in their enzyme levels in the presence of ϕ80p*su3*$^+$ (Table 9).

Since the possible effects of lysogeny on the permeability of the membrane (which may have influenced the behavior on ethionine plates) could not be ruled out, spontaneous *su*$^+$ derivatives of various *metK* mutants were isolated. Their response to ethionine was similar to that of the spontaneous *su*$^+$ derivatives of *metJ* discussed earlier. Only those with ethR phenotype suppressed to ethS in the presence of *su*$^+$ were selected for biochemical analysis. The enzyme activities of *metK su*^{0} ethR parents and their *metK su*$^+$ ethS derivatives are compared in Table 10. Strains *41a* and *42b* had *S*-adenosyltransferase levels increased in the presence of *su*$^+$, but the β-cystathionase levels remained derepressed. Ethionine-sensitive *su*$^+$ derivatives of two other *metK* mutants (*43a* and *44a*) showed no change in their enzyme activities. Thus it is not clear whether the presence of the *su*$^+$ allele is responsible for ethionine-sensitivity, nor whether it has any suppressing effect on the *metK* mutations.

S-Adenosylmethionine, the product of the reaction catalyzed by the *metK* enzyme, is involved in the biosynthesis of polyamines. Two other genes, *speA* and *speB*, are also involved in the polyamine biosynthetic pathway and map close to the *metK* gene (Maas, 1972). Maas proposed that this clustering of functionally related genes may suggest the existence of an operon concerned with spermidine biosynthesis.

TABLE 10

Specific activities of *metK* mutants and their *su*$^+$ derivatives of spontaneous origin

Strain	Relevant genotype	Relative specific activities	
		ATP:L-methionine *S*-adenosyltransferase	β-cystathionase
NR274	*metK*$^+$ ethS	1.0[a]	1.0[b]
NR274 *su*$^+$	*metK*$^+$ *su*$^+$ ethS	1.0	1.0
41a	*metK* ethR	0.22	2.67
41a su$^+$	*metK su*$^+$ ethS	0.62	3.6
42b	*metK* ethR	0.40	4.1
42b su$^+$	*metK su*$^+$ ethS	0.84	6.5
43a	*metK* ethR	0.36	2.92
43a su$^+$	*metK su*$^+$ ethS	0.36	3.75
44a	*metK* ethR	0.33	3.25
44a su$^+$	*metK su*$^+$ ethS	0.46	4.17

The enzyme activities are expressed relative to the wild type level which is taken as 1.0.

a. 9.8 μmoles *S*-adenosylmethionine formed in 20 min per mg protein.

b. Increase in absorbance at 412 nm of 0.129 per min per mg protein.

This would mean that some of the nonsense mutants defective in *metK* enzyme may not necessarily be located in the structural gene for *S*-adenosyltransferase, but could be strongly polar mutations in a neighbouring gene.

Two *amber metK* mutants (*1158* and *1168*) showing the lowest enzyme activity were combined with the polarity suppressor *suA* by transducing the *metK* strains to ilv$^+$ with P1 grown on 333*suA2* strain (see Materials and Methods). The *suA* gene is cotransductible with *ilv* with a frequency of approximately 60-80% (Morse *et al.*, 1970) but since the presence of the *suA* allele could not be identified in the transductants due to lack of suitable polar mutation, two hundred ilv$^+$ colonies were randomly purified and tested for ethionine-sensitivity. If the mutation was in the actual *metK* gene, the presence of the *suA* allele would change neither the ethionine-resistant phenotype nor the low enzyme activity. However, if it was a polar mutation next to the *metK* gene, its function might be restored and phenotype reverted to ethionine-sensitivity. Both of these mutants in a *suA* background remained ethionine-resistant indicating that the observed reduction of *S*-adenosyltransferase activity in these mutants is probably not caused by a polar mutation in an adjacent gene.

Other amber ethionine-resistant mutants

Of the twenty-four *amber* mutants isolated in strain CSH4, twenty were not cotransductible with *metB*, *serA* or *metA* loci. β-Cystathionase and *S*-adenosyltransferase levels were determined in all of them (Table 11). The majority of the mutants showed slightly

TABLE 11

β-Cystathionase and *S*-adenosyltransferase levels in CSH4-derived *amber* ethionine-resistant mutants unlinked to *metB* or *serA* loci.

Strain	Relative specific activities	
	β-cystathionase	ATP:L-methionine *S*-adenosyltransferase
wild type CSH4	1.0[a]	1.0[b]
1016	0.75	0.35
1038	0.70	0.64
1046	1.19	0.55
1060	0.70	0.38
1062	0.65	0.40
1065	3.0	1.70
1070	1.63	0.35
1113	0.55	0.46
1114	0.58	0.58
1127	0.51	0.45
1129	0.78	0.36
1130	0.66	0.41
1137	0.79	0.90
1138	0.94	0.70
1141	1.0	0.90
1146	1.20	1.30
1151	0.59	0.45
1166	0.61	0.35
1171	0.62	0.35
1174	0.62	0.34

Specific activities are expressed relative to the wild type level which is taken as 1.0.

a. Increase in absorbance at 412 nm of 0.17 per min per mg protein.

b. 77.2 nmoles *S*-adenosylmethionine formed in 30 min per mg protein.

reduced *S*-adenosyltransferase activity accompanied by a slight decrease in the levels of β-cystathionase. The enzyme activities of mutant *1065* appear to suggest a *metJ* type of mutation, whereas such activities of mutant *1070* imply a *metK* type of mutation; however, transduction mapping failed to localize these mutants in the *metJ* and *metK* genes respectively.

Resistance to ethionine could theoretically be conferred by altered or defective methylating enzymes. Since methylation is one of the major routes for methionine utilization, a deficiency or blockage in that process could lead to elevated internal pools of methionine and cause ethionine-resistance. A mutational alteration of a methylase would probably be reflected in the degree of nucleic acid methylation. In order to test this hypothesis bulk RNA was extracted from these strains and used as a substrate for methylation by an enzyme extract prepared from the wild type parent strain CSH4. As shown in Table 12, there is no indication of the presence of undermethylated RNA in any of these mutants.

Mutants defective in the transport system have been isolated by selecting analogue-resistant colonies (Schwartz *et al.*, 1959). In these strains specific permeases are altered so that they no longer transport the inhibitor into the cell. From the studies of Piperno and Oxender (1968) it appears that L-ethionine effectively competes with the methionine specific uptake system. Kadner (1974) found that ethionine did not inhibit (or only to a very small extent) the low affinity (K_T = 40 μM) methionine permease but he did not report any data with respect to the high affinity system (K_T = 0.1 μM). Therefore

TABLE 12

Methyl acceptor activity of bulk RNA from *amber* ethionine-resistant mutants unlinked to *metB* or *serA* loci

Strain	Methyl acceptor activity[a]
wild type CSH4	0.60
metB rel X341	49.20
dcm dcm GM-48	0.78
1016	0.48
1038	0.49
1046	0.58
1060	0.60
1062	0.58
1065	0.60
1070	0.40
1113	0.45
1114	0.55
1127	0.41
1129	0.56
1130	0.65
1137	0.69
1138	0.42
1141	0.60
1146	0.45
1151	0.58
1166	0.60
1171	0.60
1174	0.50

a. Methyl acceptor activity is expressed as nmoles ^{3}H-methyl group transferred in 15 min per A_{260} unit of bulk RNA.

X341 is a fully undermethylated RNA control.

GM-48 has no methyl-cystosine and only 15% methyl-adenine in DNA.

the possibility of a mutation in the transport system was examined.

Uptake of ^{14}C-methionine at a concentration of 0.8 μM by chloramphenicol-treated cells was measured at 30 sec intervals for 3 minutes. Strain 705-12, previously shown to be defective in L-ethionine uptake (unpublished observations of E. Holowachuk), was used as a control for the experimental conditions and displayed a considerably reduced rate of methionine entry (28 pmoles versus 82 pmoles taken up by wild type per 10^8 viable cells after 3 minutes of incubation). Four strains *viz.*, *metK1018*, *metK1152*, *metK1154* and ethR mutant *1038* showed significantly reduced rates of methionine uptake (Fig. 3), whereas the behaviour of the other amber mutants did not differ from the parent strain (data not presented). The transport system was fully restored when the four strains were suppressed by φ80p*su3*$^+$ (Fig. 4).

Since three of these low uptake strains have already been identified as defective in the *metK* gene product, it became apparent that they may not be true permease mutants. G. Ames (1964) pointed out that the majority of false permease mutants turn out to be excretors of the amino acid tested and she found that these are quite common among analogue-resistant strains. Kadner (1975) has also reported that both high and low affinity methionine transport systems are inhibited by increased internal pools of methionine. Consequently these low uptake mutants were tested for excretion of methionine.

The cells were grown in minimal medium under the same conditions as for previous assays (see Materials and Methods), centrifuged and the supernatant fluid from CSH4 as well as minimal medium (no methionine) used as controls. The number of pmoles of ^{14}C-methionine transported

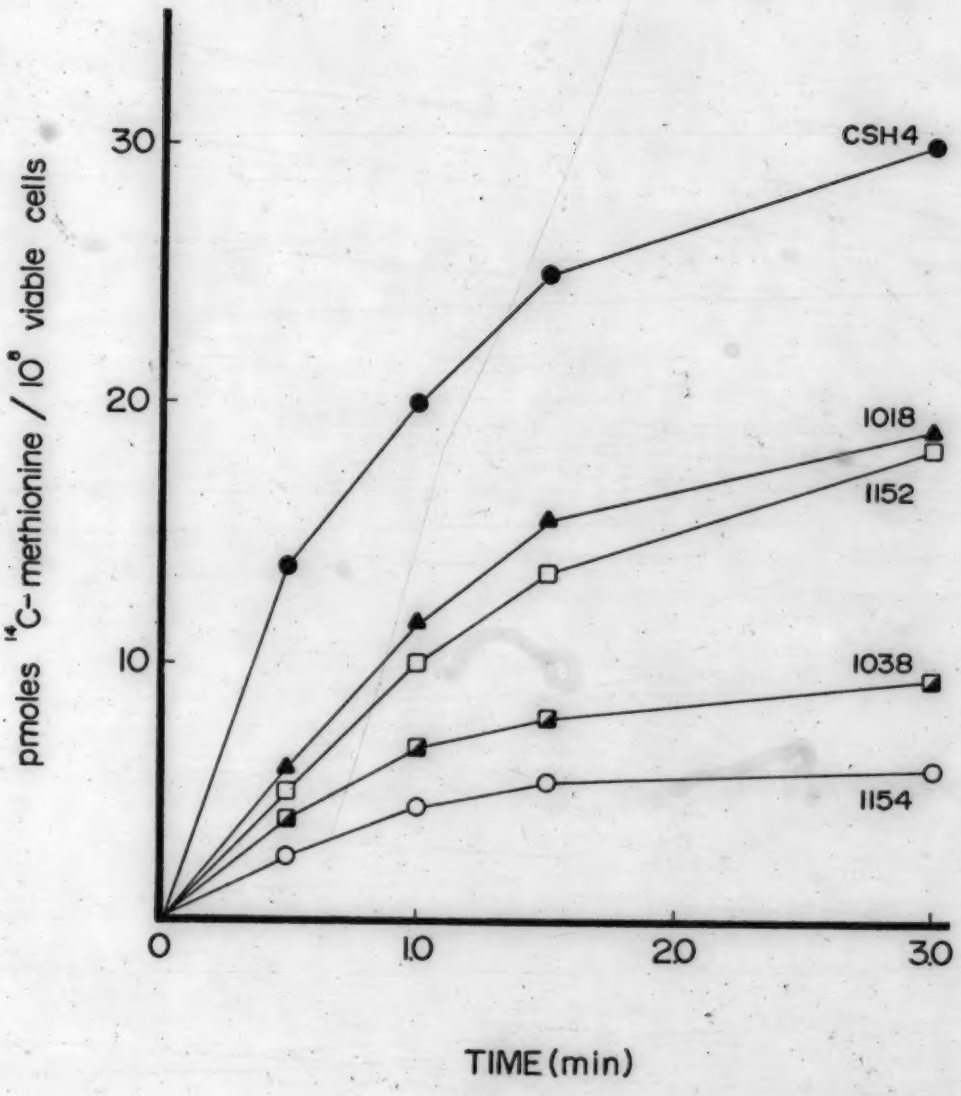

Figure 3. Comparison of ^{14}C-methionine uptake by wild type cells with the uptake by *amber metK* mutants (*1018*, *1152*, *1154*) and ethR*1038* mutant.

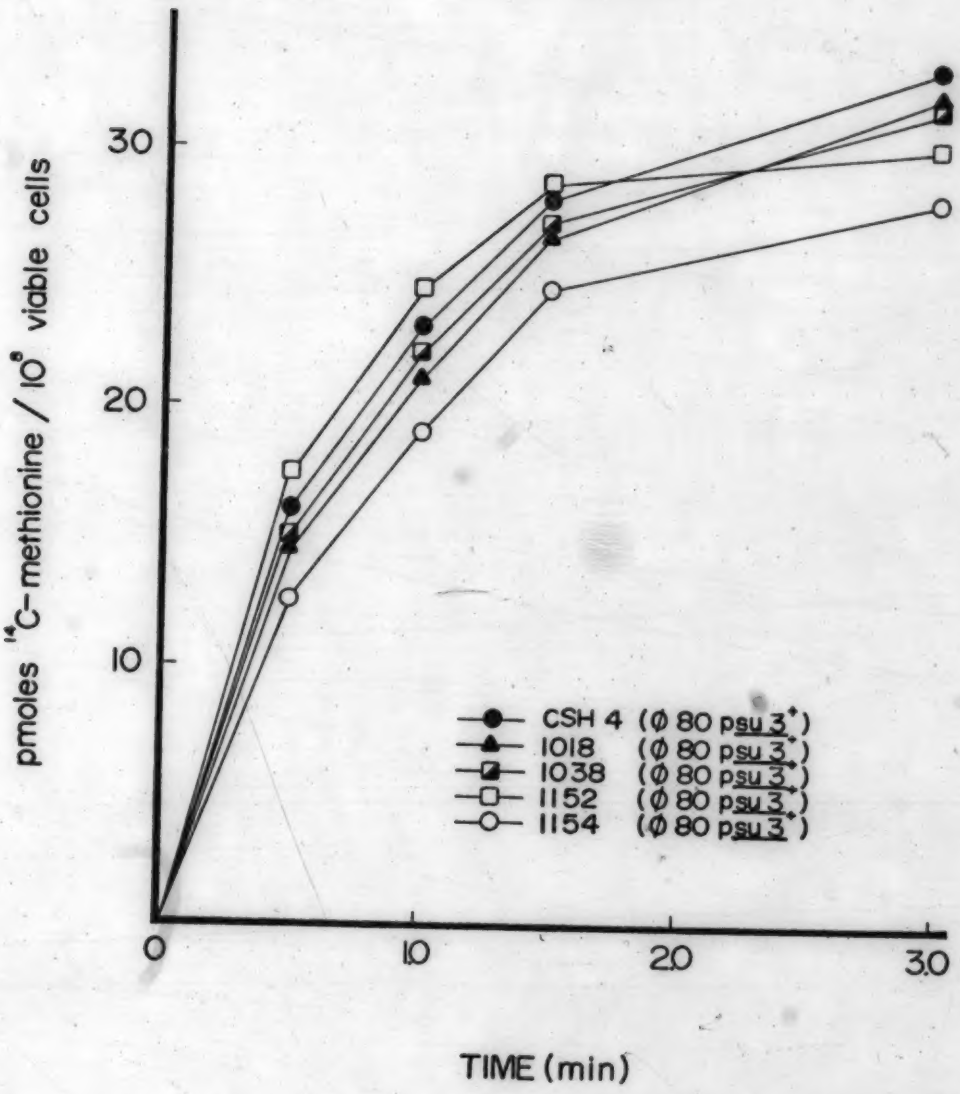

Figure 4. Time course of ^{14}C-methionine uptake by wild type cells and by *amber* mutants *viz.*, *metK1018*, *1152*, *1154*, and eth*R1038* in the presence of ϕ80p*su3*$^+$.

in 3 minutes per 10^8 viable cells for each of the supernatants tested is presented in Table 13. The inhibitory effects of the supernatant fluids from the mutants are quite dramatic and most likely due to methionine excretion. The reduced transport of ^{14}C-methionine is probably a result of dilution of the labelled material by the excreted non-labelled amino acid. As Ames (1964) points out, excretor mutants could be defective in the permease system if it was involved in retention of substrates inside the cell or in the efflux reaction.

TABLE 13

Uptake of ^{14}C-methionine by wild type CSH4 cells in the presence of culture supernatant from various ethionine-resistant mutants

Cells	Supernatant added	Methionine uptake[a]
CSH4	minimal medium	31
CSH4	CSH4	30
CSH4	*metK1018*	7.5
CSH4	*metK1152*	6.0
CSH4	*metK1154*	2.0
CSH4	ethR*1038*	5.5

Methionine uptake was measured using chloramphenicol-treated CSH4 cells to which 0.5 ml of supernatant fluid from suspected methionine excreting strains was added.

a. Methionine uptake is expressed in pmoles ^{14}C-methionine transported in 3 min per 10^8 viable cells

DISCUSSION

Spontaneous mutants of *E. coli* resistant to DL-ethionine were isolated from two strains, NR274 and CSH4, both bearing *amber* mutations in the *lac* and *trp* operons. These mutants were subjected to a screening procedure using $su3^+$ carried on ϕ80 phage and only those which contained *amber*-suppressible ethionine-resistant mutations were used for further investigation.

Lawrence *et al.* (1968) reported a distinct pattern of resistance to methionine analogues displayed by mutations in various genes involved in methionine regulation or biosynthesis. Thus in *Salmonella* the *metJ* mutants were resistant to ethionine only, the *metI* (later called *metA*) to both ethionine and α-methylmethionine, and the *metK* mutants exhibited resistance to ethionine, norleucine, and α-methylmethionine. This pattern of resistance was used as the criterium for the identification of mutants in *Salmonella*. However, when norleucine and α-methylmethionine were tested for growth inhibition of the *amber* mutants in *E. coli* no consistency was found and later classification of these mutants confirmed that the analogue-resistance pattern was unreliable for the purpose of identification. Consequently all of the *amber* mutants had to be identified by transduction mapping.

Out of the forty-one *amber* ethionine-resistant mutants isolated, ten were classified as *metJ* on the basis of transduction frequencies (92-98%) with the *metB* gene. As expected, all of these strains exhibited derepressed levels of β-cystathionase (5-14 fold) and *S*-adenosyltransferase (2-3 fold) confirming the conclusion that the *metJ* locus affects the

regulation of both these enzymes (Table 3).

Three of the *metJ amber* mutants (*65a*, *89b*, *93b*) showed complete restoration of wild type levels for four methionine enzymes (Tables 3 and 4) in the presence of *amber* suppressors. Thus the amino acid tyrosine, incorporated in response to *amber* codons in these mutants, was suppressing the mutations with high efficiency. These mutants provide clear evidence that the *metJ* gene codes for a protein involved in repression of the methionine pathway. The levels of derepression for β-cystathionase in mutants *89b* and *93b* are quite similar (relative specific activities of 11.87 and 10.55 respectively), whereas mutant *65a* is only half as derepressed.

No conclusion regarding the existence of one or more transcription units for the *metB-J-F* region can be drawn. The *metJ* gene is located in the middle of the *metB-J-F* gene cluster, and therefore it is expected that nonsense polar mutations in the *metJ* gene would produce methionine auxotrophy and as such would not be detected by the selection procedure. Thus if the *metB-J-F* region is transcribed as a unit, the *amber* mutants must be weakly polar and should map close to one end of the gene.

The original description of the *metJ* gene product being a diffusible molecule was based on the recessive character of *metJ* mutants in heterozygotes. That finding did not rule out the possibility of existence of trans-dominant constitutive mutations. Several *metJ* mutants were tested for trans-dominance, first by the ethionine inhibition test, and then by the enzyme assays. One mutant ($metJ65a_{am}$) was found to be significantly derepressed for the *metC* and *metK* enzymes in spite

of the presence of a wild type *metJ* allele (Table 6). This can be interpreted as evidence for an oligomeric structure of the *metJ* protein. The dominant *amber* mutant produces a unique polypeptide fragment. This fragment forms defective subunits that make up the repressor molecule, and when these subunits are mixed with normal ones, they are capable of inactivating the wild type polypeptides giving rise to a non-functional repressor. Another possibility is that the nonsense mutation is very close to the N-terminal sequence of the *metJ* gene and a "restart" fragment is formed which has enough subunit structure to become incorporated into the oligomeric repressor. According to this scheme, if the N-terminus is important for operator-binding activity, this hybrid repressor will be non-functional (as it is in the *lac* operon; Granem *et al.*, 1973).

The rest of the *metJ* mutants, all of which were originally classified as *ambers* on the basis of their ethionine phenotype, did not exhibit significant decrease in enzyme activities when assayed in the presence of $su3^+$ (Table 3). Most of the strains have from 10% to 40% lower β-cystathionase activity when in the suppressed state, which may account for their ethionine-sensitive phenotype. Su^+ gene is probably quite inefficient in suppressing these mutations and the degree of restored regulation reflects the efficiency of suppression. Conclusive information could have come from testing various suppressor tRNAs for better efficiency of suppression as well as from titering the ethionine-sensitivity and correlating it with the enzyme data. For instance, it is possible that efficiently suppressed mutants would display a high degree of sensitivity to ethionine as well as repressed enzyme levels,

whereas inefficiently suppressed mutants would still have considerable derepression of enzymes and be sensitive only to the highest concentration tested (3 g/l).

Among the ethionine-resistant mutants classified as *ambers* by the ϕ80p*su3*$^+$ suppression test, eleven were found to have ethionine-resistance cotransducible with the *serA* locus. All of these strains showed low *S*-adenosyltransferase activity indicating that the mutation probably affected the *metK* gene (Tables 8 and 9). Since some mutants were assayed using the TLC method and others using the BioRex-70 technique, their specific activities cannot be directly compared. In the BioRex-70 method higher concentrations of methionine were used and therefore mutants with an altered *Km* for methionine would give higher enzyme activities than by the TLC procedure. Nevertheless, the two strains with the lowest *metK* activities (*1152*, *1168*), by both methods showed only 2% or less of the wild type enzyme levels.

The internal pools of SAM have been implicated in methionine repression (Greene *et al.*, 1970) due to the fact that mutants defective in *S*-adenosyltransferase displayed elevated levels of methionine biosynthetic enzymes. However, in the case of the *amber metK1018*, *1152*, *1154* and *1168*, β-cystathionase activity was very close to normal, which is surprising since two of these mutants show almost negligible *S*-adenosyltransferase activities. A plausible explanation comes from the fact that three of these mutants were excretors of methionine (as identified by the uptake assays) and it has been demonstrated by Holloway *et al.* (1970) that β-cystathionase is usually least derepressed of all the enzymes when cells are grown in the presence of methionine. So it

appears that the *metC* enzyme may not be the best choice for determining the levels of derepression in strains which overproduce or accumulate methionine.

The results obtained using the polarity suppressor *suA* seem to suggest that the mutations which show almost no *metK* enzyme activity (*1152*, *1168*) are probably localized in the structural gene for *S*-adenosyltransferase. However, this conclusion has not been proven rigorously. One could also look for thermolability of *S*-adenosyltransferase since it is possible that the suppressed enzyme would be more heat labile than the normal enzyme indicating that the mutation occurred in the structural gene.

The existence of *amber* mutations with no *S*-adenosyltransferase activity indicates that there must be another route by which SAM is produced, otherwise these mutations would be lethal. Such a pathway has been postulated by Hobson and Smith (1973) and there is some evidence for the formation of SAM from *S*-adenosylhomocysteine in yeast (Duerre and Schlenk, 1962). If SAM is really the corepressor in methionine biosynthesis it would be necessary to assume that only the product of the *metK* gene is involved in regulation and not the SAM produced via another pathway, or possibly that pathway produces low amounts of SAM so that it is available for methylation only. On the other hand, it is conceivable that *S*-adenosylmethionine is involved in feedback inhibition of the *metA* enzyme and it is the *S*-adenosyltransferase which plays a role in repression.

Several mutants assigned to the *metJ* and *metK* classes and identified as having *amber*-suppressible ethionine phenotype did not

exhibit any change in their enzyme activities in the presence of *su3*$^+$. Spontaneous *su*$^+$ derivatives were obtained for some of these mutants and tested for ethionine-sensitivity. The ethionine-sensitive derivatives were assayed for the *metC* and *metK* enzymes, but most of them did not show a significant change in their enzyme levels in order to explain the ethionine-sensitivity. It is possible that β-cystathionase and *S*-adenosyltransferase were not the best choices and if all the other enzymes were assayed there would be one or more with repressed activity which could be rate limiting. In this case suppression would restore only partial activity of the repressor and corepressor complex so that it would recognize some but not all of the operator sites. Nevertheless, caution should be exercised when attempting to correlate the enzyme levels with the ethionine phenotype. It appears clear that this may lead to erroneous conclusions. For instance, in the case of *amber* mutants resistant to ethionine, the classification appears unreliable unless biochemical evidence is presented.

A third class of *amber* ethionine-resistant mutants was also isolated. These mutants do not belong to any previously described category. Their levels of β-cystathionase and *S*-adenosyltransferase are not significantly different from those in the wild type (Table 11) and they do not appear defective either in methionine uptake or in methylation of RNA. However, there were weaknesses in both the uptake and the methylation experiments. It is difficult to conclude that the ethionine specific transport mechanism has not been altered since only labelled methionine was tested and so far there is no direct evidence that both compounds use the same permease system. With regard to the

methylation assays, unless a gross undermethylation of one of the RNA species was present it would probably not be detected using bulk RNA preparations. One of the unclassified mutants (*1038*) was identified as an excretor of methionine when tested for defective methionine uptake (Table 13). The excretion of methionine in this mutant is probably responsible for its ethionine-resistant phenotype. It is possible that all of the other unclassified mutants had elevated pools of methionine (although they did not excrete it) and therefore acquired ethionine-resistance. On the other hand, these strains may have a more efficient degradation mechanism for ethionine than the wild type cells.

In conclusion, these studies demonstrate that (1) the *metJ* gene codes for a repressor protein, most likely oligomeric, which regulates the expression of the methionine genes, (2) this repressor probably interacts with *S*-adenosylmethionine which is normally produced by the *metK* gene, (3) there may be an alternative pathway for *S*-adenosyl-methionine production or a different methyl donor may be utilized, and (4) an additional class of mutants exists in which ethionine-resistance is conferred by a mechanism that may not be directly related to repression.

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